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(54) Title: LFA-3 AS A VACCINE ADJUVANT

(57) Abstract

Vaccines comprising an immunogen and, as an adjuvant, LFA-3 or a fragment of LFA-3 capable of binding to CD2, a surface receptor on T-lymphocytes, are disclosed. LFA-3 has been found to markedly enhance the proliferation of activated T-cells and thus is capable of augmenting the immune response. Methods of vaccination in which LFA-3 is employed in conjunction with an immunogenic inoculum are also disclosed.

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LFA-3 AS A VACCINE ADJUVANT

TECHNICAL FIELD OF THE INVENTION

This invention relates to the use of lymphocyte function associated antigen-3 (LFA-3) as an adjuvant in a vaccine mixture. LFA-3 is capable of augmenting T-lymphocyte proliferation and thus enhancing the immune response to a vaccine's immunogenic component.

BACKGROUND OF THE INVENTION

The immune system has evolved primarily to combat infection by pathogenic organisms. To accomplish this, the immune system has highly specialized effector components and complex regulatory mechanisms. The immune response is not confined to a single site in the body; effector cells move within and among lymphoid organs and various body compartments. On a cellular level, the immune response to a pathogen depends on a complex system of communication between the various cell types of the immune system. The mechanism of this communication among the various cells may be either by direct cell-to-cell contact or by soluble secreted mediators (cytokines) which can act at a distance from the cell that secretes them.

There are generally two facets of the immune response: a <u>cell mediated response</u>, primarily

comprising the action of cytotoxic T cells which attack and kill foreign cells or virus-infected cells, and a humoral response, comprising the activation of B cells to plasma cells which secrete antibodies specific for foreign macromolecules.

T cells play a central role in both types of responses (1) by interacting directly with target cells in the production of cytotoxic T cell-mediated responses and (2) by interacting with antigen-presenting cells (APCs) bearing foreign macromolecules in the initiation of antibody responses. In the latter situation, when the APCs are B cells, the B cells are activated to plasma cells which secrete antibodies of pre-determined specificity. A specialized subset of T cells called helper T cells plays a unique role in increasing the specificity of the B cell antibody response and in the development of "memory" B cells, which are critical for anamnestic or secondary responses to a renewed challenge by a foreign antigen.

The interaction of T cells with target and antigen-presenting cells is highly specific and depends on the recognition of a surface antigen on the target cell or APC (or B cell) by highly specific receptors on the T cells. This interaction may be facilitated by other antigens expressed on the surface of the interacting cells, e.g., the antigen receptor complex known as CD3 on T cells or other T cell accessory molecules such as CD4, LFA-1, CD8 and CD2, and accessory molecules such as LFA-3, ICAM-1 and MHC on antigen-presenting cells. Interaction between accessory molecules on T-lymphocytes and accessory molecules on target cells or APCs (or B cells) may be important in mediating intercellular adhesion and are thought to enhance the efficiency of lymphocyte/APC and lymphocyte/target cell interactions. In this way, the

interaction of accessory molecules is important in mediating cytotoxic T-lymphocyte killing of target cells and T cell-mediated immune responses (including antibody responses) in general.

For example, recent studies have shown that there is a specific interaction between CD2 (a T-lymphocyte accessory molecule) and LFA-3 (a target cell accessory molecule) that mediates T-lymphocyte adhesion to the target cell. This adhesion is essential to the initiation of the T-lymphocyte functional response (M. L. Dustin et al, "Purified Lymphocyte Function-Associated Antigen-3 Binds To CD2 And Mediates T-Lymphocyte Adhesion," J. Exp. Med., 165, pp. 677-92 (1987); Springer, et al, "The Lymphocyte Function Associated LFA-1, CD2, and LFA-3 Molecules: Cell Adhesion Receptors Of The Immune System," Ann. Rev. Immun., 5, pp. 223-52 (1987)). In addition, purified phosphoinositol-linked LFA-3 (PI-LFA-3) in multimeric, micellar form has been shown to bind to CD2 on T-lymphocytes and to initiate T-lymphocyte proliferation in vitro (M. L. Dustin et al, "Correlation of CD2 Binding and Functional Properties of Multimeric and Monomeric Lymphocyte Function-Associated Antigen 3," J. Exp. Med., 169, pp. 503-17 (1989)).

Classical vaccine compositions are designed to activate the immune system in order to confer immunity, prophylactically, to subsequent challenges from a living pathogen that might infect and debilitate an individual or animal. Vaccines generally contain two main components. The first is an immunogen which the host's immune system will recognize as foreign (not self). The immunogen is usually an attenuated viral or bacterial pathogen or proteins or polypeptides derived from the pathogen. The second component is an

adjuvant. Classical adjuvants have ranged from simple metal salts, e.g., $Al(OH)_3$ (known as Alum), to complex emulsified mixtures such as Freund's adjuvant, a water-in-oil emulsion containing suspended tubercle bacilli. Adjuvants act primarily by creating a depot from which immunogen is slowly released, thereby imitating the persistent challenge posed to the immune system during an infection by a functional replicating organism or virus. Adjuvants may also contain a plethora of foreign biomolecules (e.g., tubercle bacilli) which strongly sensitize the cells of the immune system to both the adjuvant biomolecules themselves and the included immunogen. In this regard, adjuvants enhance the immunogenicity of a particular immunogen.

Previously used adjuvants have various shortcomings. Many adjuvants cause inflammation at the site of administration, resulting in discomfort. Adjuvants derived from inactivated viral or bacterial sources may also be toxic and, consequently, of limited usefulness despite their high potency. Additionally, conventional vaccines are indiscriminate in their excitement of the immune system, recruiting into action B-lymphocytes, monocytes and neutrophils as well as T-lymphocytes. Accordingly, there is a need for new vaccines that are less likely to cause inflammation, are less toxic, and target the response of the immune system to cells (i.e., T-lymphocytes and B cells) that are directed to a particular antigen.

SUMMARY OF THE INVENTION

This invention addresses the shortcomings of such conventional adjuvants. One aspect of this invention is a composition comprising LFA-3 and an immunogen to form a vaccine mixture. Both the adjuvant and the immunogen are present in amounts sufficient to

stimulate and augment the immune response in a host animal toward the immunogen. Another aspect of this invention is a method of vaccination comprising administration of an LFA-3 adjuvant/immunogen mixture to a host animal. A particularly preferred embodiment of the present invention is a vaccine comprising an immunogen and LFA-3 as an adjuvant, and also a co-adjuvant comprising a ligand or an antibody recognizing other T-cell surface molecules than CD2 (e.g., CD3), or the same or a different epitope of CD2 than that bound by LFA-3 (i.e., $T11_1$). Examples of antibodies recognizing one of the epitopes of CD2 include anti-$T11_1$, anti-$T11_2$ or anti-$T11_3$, 9.6, CD2.1, 9-1, 35.1, D66, and GT2. The compositions and methods of the present invention substantially avoid irritation and inflammation at the site of administration as well as toxic side effects resulting from the adjuvant. Furthermore, the use of LFA-3 in the presence of an immunogen dramatically increases proliferation of T-lymphocytes, thereby enhancing the cell-mediated immune response to an immunogen, increasing the vaccine's effectiveness.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing levels of immunoglobulin secretion by monocyte-depleted human peripheral blood lymphocytes (PBLs) subjected to different stimuli. The lymphocytes were cultured for 7 days in the presence of a fixed amount of anti-$T11_2$ alone, different concentrations of PI-LFA-3 alone, or a combination of a fixed amount of anti-$T11_2$ and different concentrations of PI-LFA-3.

Figure 2 is a graph showing level of immunoglobulin secretion by monocyte-depleted human peripheral blood lymphocytes subjected to different

stimuli. The lymphocytes were cultured for 7 days in the presence of PI-LFA-3 alone (10 μg/ml), different concentrations of anti-$T11_2$ alone, or a combination of a fixed amount of PI-LFA-3 (10 μg/ml) and different concentrations of anti-$T11_2$ or a murine myeloma immunoglobulin of the same isotype (IgG_{2a}).

DETAILED DESCRIPTION OF THE INVENTION

Any source and form of LFA-3 is suitable provided that the LFA-3 employed is compatible with the host animal. For example, human LFA-3 should be used as the adjuvant in vaccines according to this invention intended for use in humans. However, this invention contemplates cross-species use of LFA-3 where cross-reactivity is observed without adverse side effects. For example, if, e.g., primate LFA-3 is determined to be active in humans and does not have any serious side-effects, this cross-species use is within the concept of the invention. The form of LFA-3 employed should itself be of limited toxicity or immunogenicity to the host. Preferably, the LFA-3 is either the complete LFA-3 molecule or a fragment capable of binding to CD2. More preferably, the LFA-3 will be the form having a carboxyterminal phophatidylinositol linkage (PI-LFA-3), discussed completely in copending, commonly assigned U.S. application Ser. No. 237,309, filed August 26, 1988, incorporated herein by reference. Even more preferably, the LFA-3 will be in multimeric micellar form. Most preferably, the LFA-3 is PI-LFA-3 in an octameric, micellar form.

The LFA-3 may be in any form that leads to a sufficiently high binding affinity which, in turn, enhances activated T-lymphocyte proliferation or leads to crosslinking of receptor sites on the T-lymphocyte cell surface.

Minor variations of the primary amino acid sequence of LFA-3 may occur and are within the scope of this invention, so long as the derivative LFA-3 binds to the CD2 receptor. Furthermore, amino acid site-specific mutations, non-naturally-occurring amino acids, peptide structural analogs or phosphoinositol analogs may be used to augment the LFA-3 or to replace a portion of the LFA-3 molecule. Soluble forms of LFA-3 may also be used. These modifications may be used to improve the biological stability of the LFA-3 adjuvant or to alter the structure of LFA-3 micelles formed from LFA-3 molecules. For example, a different hydrophobic moiety may be used to replace the carboxy-terminal phosphoinositol appendage of PI-LFA-3. Alternatively, an LFA-3 derivative having a hydrophobic moiety attached to the LFA-3, preferably at its carboxy-terminal end, may form a multivalent micelle. The stoichiometry of such micelles, however, will depend upon the hydrophobic moiety used as well as the particular LFA-3 derivative. Suitable alternative hydrophobic moieties include, e.g., phosphatidylethanolamine.

Alternatively, the LFA-3 may be in the form of conjugates, such as fusion proteins, coupled proteins and immunoconjugates. For example, using recombinant techniques, the LFA-3 may be expressed as a fusion protein linked to an immunogen, a co-adjuvant, or a protein sequence which causes aggregation of LFA-3, e.g., domains of C4 binding protein or the amino acid sequence of gelsolin which binds to phosphoinositol. Also, LFA-3 may be chemically coupled in known ways directly to an immunogen, providing a single molecule with dual functionality with respect to the immune system. LFA-3 linked to an immunogen by recombinant or chemical techniques would be expected to

significantly enhance the antibody response to that immunogen by stimulating a productive cell-to-cell interaction between an immunogen-specific B cell and any T cell stimulated by the LFA-3 linked to that immunogen. The LFA-3 may also be in the form of an immunoconjugate when coupled to antibodies, e.g., where anti-$T11_3$ or anti-CD3 antibodies are coupled with LFA-3. Mixtures of LFA-3 conjugates may also be used. Regardless of the structure used, the LFA-3 should survive in vivo for a sufficient time to augment lymphocyte activation.

LFA-3 may be obtained in substantially pure form from either native or recombinant sources. For example, the PI-LFA-3 may be isolated, purified, and converted to micellar form following methods described above (Springer, 1989). A selection may be made among commonly used isolation and purification procedures without departing from the scope of this invention. Plasmids bearing genes coding for recombinant LFA-3 and recombinant PI-linked LFA-3 have been deposited with In Vitro International under the terms of the Budapest Treaty, under accession nos. IVI-10133 and IVI-10180.

The immunogen may be any biological system capable of eliciting an immune response against the immunogen. The immunogen may be derived from either natural, recombinant, or synthetic sources or mixtures thereof; furthermore, it may be used in substantially pure form or it may be a crude mixture of biomolecules. Immunogens may be derived from viral or bacterial sources. For example, immunogens may be derived from agents responsible for acne vulgaris, caries, cholera, gonorrhea, haemophilus, klebsiella, lactobacillus, leprosy, measles, meningitis, otitis media, pertussis, rubella, syphilis, shigella, malaria, hoof and mouth disease, adenovirus, AIDS, HTLV, CMV, dengue, GBV,

herpes simplex, hepatitis A, hepatitis B, hepatitis non A-non B, hepatitis C, influenza, lassa fever, parainfluenza, pneumonia, parvovirus, rotavirus, or RSV. Bacterial or viral sources of the immunogen generally are inactivated or attenuated by heat or chemical treatment.

The amounts of the adjuvant and immunogen needed to evoke an immune response in the host are interrelated, but are within the ranges generally employed in conventional vaccines. For example, the use of increasing amounts of adjuvant may suggest that decreasing amounts of immunogen can be used, and vice versa. The preferred amount of adjuvant is between about 0.05 microgram and about 5.0 milligrams per dose. More preferably, between about 0.8 microgram and about 2.0 milligrams is used. The preferred amount of immunogen is between about 0.05 microgram and about 5 milligrams per dose. More preferably, between about 0.8 micrograms and about 2 milligrams of the immunogen is used. Those skilled in the art will understand that the dosage will depend upon the host receiving the vaccine as well as its size, weight, metabolism, etc. Higher doses of the immunogen may produce side effects such as chills, fever, and the like. Higher doses of the immunogen are within the scope of this invention should the clinical advantage outweigh these side effects.

The compositions of this invention may be formulated using methods and compositions similar to those used for other pharmaceutically important polypeptides. Thus, the adjuvant and immunogen may be stored in lyophilized form and reconstituted in a physiologically acceptable vehicle prior to administration. Alternatively, the adjuvant and immunogen may be stored in the vehicle. Preferred

vehicles are sterile solutions. Preferred vehicles include sterile buffer solutions, such as phosphate buffered saline. Any method of combining the adjuvant and the immunogen in the vehicle that retains the immunoreactivity of the mixture is appropriate.

The vehicle may contain preservatives or other known additives which are used to improve the shelf stability or the efficacy of the mixture. Suitable preservatives include e.g., thimerosal.

The vaccine mixture also may contain additional materials that supplement the ability of LFA-3 to stimulate lymphocyte activation. For example, the combination of LFA-3 and a co-adjuvant reactive with surface molecules of T-lymphocytes (or other cells) may be used. The immune system of a host is stimulated and reacts against the vaccine of this invention very rapidly. Thus, a vaccine mixture containing LFA-3 and optionally a co-adjuvant may be used in a therapeutic manner. Suitable co-adjuvants may be other surface molecules of cells involved in the immune response or fragments thereof that recognize such molecules, antibodies to such cell surface molecules (including V_H and F_V antibody fragments that bind to surface antigens) or mixtures thereof. Preferably, these additional materials are antibodies which recognize T-cell surface molecules, most preferably antibodies which recognize the same or a different epitope of CD2 than recognized by LFA-3. Most preferably, the co-adjuvant will be an antibody recognizing the $T11_1$ epitope, the same CD2 epitope recognized by LFA-3 (e.g., anti-$T11_1$); or an antibody recognizing the $T11_2$ epitope or the $T11_3$ epitope (e.g., anti-$T11_2$ or anti-$T11_3$), which recognize different CD2 epitopes than recognized by LFA-3; or combinations thereof.

The volume of a single dose of the vaccine of this invention may vary but will be generally within the ranges commonly employed in conventional vaccines. The volume of a single dose is preferably between about 0.1 ml and about 1.0 ml, more preferably between about 0.2 ml and about 0.5 ml.

The adjuvant/immunogen mixture may be administered by any convenient means. Preferred methods of administration include subcutaneous, intraperitoneal, intramuscular, or intravenous injection. Alternatively, the mixture may be released from a biodiffusible implant. A single administration may be used, or preferably a series of administrations may be made over the course of several days or weeks.

The following examples are intended to illustrate the invention but are not to be construed as limiting the same.

EXAMPLE 1

Multimeric PI-LFA-3 was isolated in the following manner. CHO cells, transfected with LFA-3 cDNA coding for the phosphoinositol linked form of LFA-3 (PI-LFA-3) were grown on collagen beads in roller bottles to 1.2×10^7 cells/ml. 150 ml of cell coated beads in DMEM medium were treated with 50 mg collagenase for 30 min at 37°C. Cells were pelleted, washed 2 times in 100 ml conditioned medium, then in 50 ml 1x PBS, followed by 50 ml Dulbecco's medium. Cell pellets were incubated in 20 ml of PBS; 2% NP40; 1 unit/ml trypsin inhibitor; 1 mM PMSF for 2 hrs at 4°C. Cells were then pelleted at 40,000 rpm in a 70.1 Ti-Beckmann ultracentrifugation rotor for 75 min at 4°C. Supernatant was applied to a TS2/9 affinity column, essentially as described by Dustin et al, J. Exp. Med., 169, pp. 503-517 (1989).

We assessed the ability of PI-LFA-3 to augment T-lymphocyte production in the following manner. Whole human blood, drawn by venipuncture, was overlaid onto Lymphocyte Separating Medium (ORGANON TEKNIKA) to separate peripheral blood lymphocytes (PBLs) from plasma, red blood cells, and polymorphonuclear cells. The PBLs were taken up in RPMI-1640 (GIBCO). The growth medium was made to be 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units penicillin G/ml growth medium, 100 micrograms streptomycin G/ml growth medium, and 5.5×10^{-5} M 2-mercaptoethanol.

The cells suspended in growth medium were placed on tissue culture treated plastic dishes (COSTAR®; Cambridge, MA); the cells were incubated for two cycles of 1 hr per cycle at 37°C to adhere the monocytes to the dishes. T-lymphocytes and B-lymphocytes, still suspended in solution, were removed by pipetting and transferred to the flat bottom wells of a 96-well tissue culture plate. 1×10^5 cells in 50 microliters growth medium were added to each well.

The 96 wells (all containing suspended cells) were grouped to receive phorbol myristate acetate (PMA), anti-$T11_2$ (a gift of E. Reinherz, Dana-Farber Cancer Institute, Boston, MA), PI-LFA-3, various combinations of these three additives, or none of these additives (control). Thus, an equal number of wells comprised, in addition to the cells, no additive (control), PMA, anti-$T11_2$, PI-LFA-3, PMA and anti-$T11_2$, PMA and PI-LFA-3, PI-LFA-3 and anti-$T11_2$, and PMA, anti-$T11_2$ and PI-LFA-3.

For wells receiving PMA, 50 microliters of a stock solution of phorbol myristate acetate (PMA) in growth medium was added so that the final concentration of PMA in the well was 0.6 ng/ml.

For wells receiving anti-$T11_2$, 50 microliters of a stock solution containing anti-$T11_2$ in growth medium was used to obtain a final dilution in anti-$T11_2$-containing wells of 1:1000. The antibody stock solution was prepared by diluting 1:250 ascites drawn from a mouse injected intraperitoneally with a hybridoma that produces anti-$T11_2$. The ascites drawn directly contained approximately 1 mg/ml of anti-$T11_2$.

For wells receiving PI-LFA-3, 50 microliters of micellar PI-LFA-3 in growth medium was then added to the well. Varying concentrations of PI-LFA-3 were tested, i.e., 3 ng/ml, 30 ng/ml and 300 ng/ml.

The total volume of each well was 200 microliters. If PMA, anti-$T11_2$ or PI-LFA-3 was omitted from a particular well, then sufficient growth medium was added to make the total volume 200 microliters.

The solutions were incubated for 66 hours at 37°C. At that time, 50 microliters of medium containing 1 microCurie [^{3}H]-thymidine was added to each well and the solutions were incubated for a further 6 hours at 37°C.

The contents of the wells were washed repeatedly in a SKATRON harvester (SKATRON, Inc.; Sterling, Virginia), which automatically lysed the cells and collected the cellular DNA on filter paper, which filter paper, in turn, was read in a SKATRON Betaplate Scintillation Counter. Proliferation of T-lymphocytes was directly measured by detecting the incorporation of [^{3}H]-thymidine.

The results of four trials are shown in the table below:

	counts per minute (cpm)			
	Trial 1	Trial 2	Trial 3	Trial 4
medium control	94	532	97	290
PMA only	2,517	1,449	238	1,872
PMA + α-T11$_2$	--	--	--	2,674
α-T11$_2$	109	428	43	291
PMA + PI-LFA-3	1,669	--	168	469
PI-LFA-3	--	--	--	315
PI-LFA-3 + α-T11$_2$	1,023	--	168	1,690
PMA + α-T11$_2$ + PI-LFA-3				
300 ng/ml PI-LFA-3	133,921	238,633	197,689	170,306
30 ng/ml PI-LFA-3	123,227	195,775	122,267	157,318
3 ng/ml PI-LFA-3	71,894	34,011	1,017	--

The maximum T-lymphocyte proliferation as measured in counts per minute, occurred when PMA, anti-T11$_2$ and PI-LFA-3 were together. The threshold of augmentation of T-lymphocytes by PI-LFA-3 occurred at a concentration of 30 ng/ml. At this concentration, an average number of counts per minute due to paper-adhered [^{3}H]-thymidine was 195,779. The number of counts obtained from a control experiment identical to that described except that PI-LFA-3 was omitted was 2000 counts per minute.

EXAMPLE 2

The following example investigates T-lymphocyte proliferation in the presence of anti-T11$_3$, anti-T11$_2$ (gifts of E. Reinherz, Dana-Farber Cancer Institute, Boston, MA), PI-LFA-3, and the various combinations thereof.

PBLs were isolated as in Example 1, except that the PBLs were not incubated in tissue culture plastic treated dishes. The cells were taken up in growth medium, and 1 x 10^5 cells in 50 microliters

growth medium added to each well of a 96-well U bottom tissue culture plate. Test solutions were added to specific wells in 50 microliter aliquots to give the indicated final concentration. Control wells contained medium and PBLs only. Test wells contained a combination of anti-CD2 monoclonal antibodies anti-$T11_2$ and anti-$T11_3$ at a final dilution of 1:900 of ascites; or anti-$T11_2$ with PI-LFA-3; or anti-$T11_3$ with PI-LFA-3; or each compound individually. PI-LFA-3 (prepared as in Example 1) was added at increasing concentrations ranging from 0.015 to 0.5 microgram/ml. All wells were brought to a final volume of 150 microliters with growth medium. Cells were incubated at 37°C for 3 days, after which 50 microliters of 20 microCurie/ml [^{3}H]-thymidine was added and the cells incubated for 12 hrs at 37°C. At this point, the cells were lysed and harvested in a manner similar to Example 1. Proliferation of T-lymphocytes was directly measured by detecting the incorporation of [^{3}H]-thymidine. Results are presented in the following table:

	counts per minute ($\times 10^{-3}$)			
PI-LFA-3, microgram/ml	with α-$T11_{2+3}$	with α-$T11_3$	with α-$T11_2$	with buffer
0.500	401	320	19	4
0.250	375	240	13	4
0.125	413	196	11	6
0.060	360	134	8	5
0.030	368	68	9	6
0.015	290	29	7	6

Medium control	9,000 cpm
α-$T11_{2+3}$ + buffer	270,000 cpm
α-$T11_3$ or α-$T11_2$ + buffer	5,000 cpm

The results showed that a significant increase in T-lymphocyte proliferation occurred when

PI-LFA-3 was used in combination with anti-$T11_3$, or a combination of both anti-$T11_3$ and anti-$T11_2$.

EXAMPLE 3

The following example demonstrates T cell-dependent B cell activation to antibody secretion in the presence of anti-$T11_2$ and PI-LFA-3. (See Figures 1 and 2.)

PBLs were isolated from buffy coat cells purchased from the American Red Cross (Northeast Regional Blood Services) by the method described in Example 1. After isolation from a lymphocyte separating medium (Ficoll-Paque; Pharmacia, Piscataway, NJ), removal of the monocytes and washing, the cells were taken up in growth medium, and 2×10^5 cells in 50 microliters growth medium were added to each well of a 96-well U-bottom tissue culture plate. Positive control wells received no additions (other than growth medium to compensate for volume on a like basis for additions to the test wells). Test wells in one experiment (see Figure 1) contained a constant amount of anti-$T11_2$ antibody (final dilution 1:900 of ascites) alone or in combination with varying amounts of PI-LFA-3. Test wells in another experiment (see Figure 2) contained a constant amount of PI-LFA-3 (10 μg/ml) alone or in combination with varying amounts of anti-$T11_2$ antibody. Negative control wells contained a murine myeloma immunoglobulin of an isotype identical to that of the anti-$T11_2$ antibody (IgG_{2a}), either alone or in combination with PI-LFA-3 (10 μg/ml). All additions were dissolved and/or diluted in growth medium. After all of the additions, each culture well contained 200 μl of growth medium. Cells were incubated 37°C, in a 5% CO_2 atmosphere for seven days. After seven days, 100 microliters of conditioned

culture medium supernatant (i.e., devoid of cells) was removed; triplicates were pooled and held at -20°C until assayed. The conditioned medium was assayed for human immunoglobulin of the G and M classes by incubating said medium on microtiter ELISA plates coated with a purified goat antibody raised to human IgG and IgM (purchased from Jackson Immunoresearch, Malvern, PA). Human immunoglobulin present in the conditioned culture medium that bound to the goat anti-human IgG and IgM coated ELISA plates was detected with an alkaline phosphatase conjugate of goat anti-human IgG and IgM. The bound enzyme was illuminated by conversion of an uncolored substrate to a colored product. Color development, which directly correlates to the amount of human Ig in the culture supernatant, was quantified on a Molecular Devices Thermomax ELISA reader at a wavelength of 405 nm.

Cultures of purified T and B cells in the absence of monocytes secreted immunoglobulin during the seven day culture period. In the absence of any additions, this level of Ig secretion is referred to as the "100% control" level, and the amount of Ig in each of the test cultures on that plate is related to this 100% control (no additive) level. The results depicted in Figure 1 indicate that monocyte-depleted PBLs cultured in the presence of a constant level of anti-$T11_2$ antibody and varying amounts PI-LFA-3 show significant enhancement of Ig production. The results depicted in Figure 2 indicate that monocyte-depleted PBLs cultured in the presence of a constant level of PI-LFA-3 and varying amounts of anti-$T11_2$ antibody show significant enhancement of Ig production. These results confirm the adjuvant nature of a composition containing PI-LFA-3 for T cell-dependent B cell activation to immunoglobulin secretion.

EXAMPLE 4

Vaccine mixtures containing human LFA-3 and hepatitis B core antigen are tested to investigate the effect of LFA-3 as an adjuvant in an *in vivo* model. Vaccine mixtures containing 0.01-1.0 mg micellar PI-LFA-3 and 0.01-1.0 mg hepatitis B core antigen (Biogen, Inc., Cambridge, MA) are injected intramuscularly into rhesus monkeys three times at monthly intervals. Control groups of rhesus monkeys are injected with PI-LFA-3 alone, hepatitis B core antigen alone and buffer alone. Whole blood is drawn from the monkeys before immunization and at weekly intervals during the course of immunization and at monthly intervals thereafter to a year after immunization. Serum is removed from clotted whole blood and the presence of antibodies to the hepatitis B core antigen confirmed using an ELISA assay. Adjuvant activity of PI-LFA-3 is demonstrated by either greater anti-hepatitis B core antigen antibody titers or by a more prolonged presence of such antibodies in serum from monkeys receiving both PI-LFA-3 and hepatitis B core antigen when compared to serum titers obtained from monkeys of the control groups. To assess cellular immune function in the immunized monkeys, PBLs are isolated from heparinized whole blood in a manner similar to Example 1 and proliferation of activated T-lymphocytes is assayed by culturing PBLs with 0.001-0.1 mg hepatitis B core antigen with and without PI-LFA-3 under standard tissue culture conditions in microliter tissue culture plates. Proliferation is measured by incorporation of $[^3H]$-thymidine after 3-5 days of culture as in Example 1. Evidence of adjuvant efficacy is demonstrated by augmented proliferation of T-lymphocytes from rhesus monkeys receiving core antigen and PI-LFA-3, compared

against T-lymphocyte proliferation in monkeys of the control groups.

EXAMPLE 5

Human CD2 (h-CD2) transgenic mice (a gift from Dimitri Kioussis, Mill Hill, UK) are used as a model system to determine the ability of PI-LFA-3 to augment T-lymphocyte activation *in vivo*. Human CD2 has been shown to transduce T-cell activation signals to murine T-cells in the same manner as to human T-cells. For this experiment, h-CD2-transgenic mice are injected with increasing amounts of PI-LFA-3 and ovalbumin; h-CD2-transgenic mice in one control group are injected with ovalbumin alone. A second group of control h-CD2-transgenic mice are injected with PI-LFA-3 alone. 0.1-8 micrograms PI-LFA-3 and 0.1-100 micrograms ovalbumin in 0.1 ml sterile PBS to form increasing doses are injected subcutaneously into h-CD2-transgenic mice. Control mice are injected with 0.1-100 micrograms ovalbumin in 0.1 ml sterile PBS or with 0.1-8 micrograms PI-LFA-3 in 0.1 ml sterile PBS. As further controls for LFA-3 activity on h-CD2-transgenic mice, groups of non-transgenic (normal) mice are immunized with the same moieties listed above. After 8-14 days, to assess cellular immunity, the draining lymph nodes are removed and a single cell suspension of lymphocytes is isolated. The lymphocytes are cultured in a growth medium of RPMI-1640 made to be 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units penicillin G/ml growth medium, 100 micrograms stretomycin G/ml growth medium, and 5.5×10^{-5} M 2-mercaptoethanol. The cells are transferred to flat bottom wells of a 96-well tissue culture plate. 2×10^{5} cells are added to each well in 50 microliters of growth medium.

The wells are grouped to receive increasing amounts of ovalbumin ranging from 0.1 microgram to 100 micrograms per milliliter, and the final volume of the wells is made to be 200 microliters. The lymphocytes are incubated for 2-5 days, at which time 50 microliters of growth medium containing 1 microCurie/ml of [^{3}H]-thymidine are added to each well. The cultured lymphocytes are incubated for 6 hours more. The cells are harvested and [^{3}H]-thymidine incorporation measured as described in Example 1.

In addition, anti-ovalbumin antibody responses are determined using conventional ELISA techniques. Mice are bled prior to immunization and two weeks following immunization; the blood is allowed to clot and serial dilutions of the aspirated serum are assayed for anti-ovalbuim activity.

We expect to find that PI-LFA-3/ovalbumin injected into mice augments anti-ovalbumin immune responses significantly over mice injected with ovalbumin alone.

WE CLAIM:

1. A vaccine comprising (1) an immunogenic component and (2) an adjuvant component, wherein the adjuvant comprises LFA-3 or a fragment thereof capable of binding to CD2.

2. The vaccine of claim 1, wherein the LFA-3 is PI-LFA-3.

3. The vaccine of claim 2, wherein the PI-LFA-3 is in multimeric form.

4. The vaccine of claim 3, wherein the LFA-3 is in the form of a micelle.

5. The vaccine of claim 1, wherein the LFA-3 is in the form of a conjugate.

6. The vaccine of claim 1, wherein the immunogenic component includes a composition selected from attenuated agents responsible for diseases of the group consisting of acne vulgaris, caries, cholera, gonorrhea, haemophilus, klebsiella, lactobacillus, leprosy, measles, meningitis, otitis media, pertussis, rubella, syphilis, shigella, malaria, hoof and mouth disease, adenovirus, AIDS, HTLV, CMV, dengue, GBV, herpes simplex, hepatitis A, hepatitis B, hepatitis non A-non-B, hepatitis C, influenza, lassa fever, parainfluenza, pneumonia, parvovirus, rotavirus, and RSV.

7. The vaccine of claim 1, wherein the LFA-3 component and the immunogenic component are coupled.

8. The vaccine of claim 1, which further contains a co-adjuvant consisting of a polypeptide or antibody that binds to a surface antigen on T-lymphocytes.

9. The vaccine of claim 7, wherein the co-adjuvant is anti-$T11_1$, anti-$T11_2$ or anti-$T11_3$, or fragments thereof recognizing an epitope of CD2.

10. A method of vaccination comprising inoculating a mammal with (1) an immunogen capable of conferring on said mammal immunity against a disease and (2) an amount of an adjuvant consisting essentially of LFA-3 or a fragment thereof capable of binding to CD2 effective to enhance the mammal's immune response to said immunogen.

11. The method of claim 9, wherein the LFA-3 is PI-LFA-3.

12. The method of claim 10, wherein the PI-LFA-3 is in multimeric form.

13. The method of claim 11, wherein the LFA-3 is in the form of a micelle.

14. The method of claim 9, wherein the LFA-3 is in the form of a conjugate.

15. The method of claim 9, wherein the immunogenic component includes a composition selected from attenuated agents responsible for diseases of the group consisting of acne vulgaris, caries, cholera, gonorrhea, haemophilus, klebsiella, lactobacillus, leprosy, measles, meningitis, otitis media, pertussis,

rubella, syphilis, shigella, malaria, hoof and mouth disease, adenovirus, AIDS, HTLV, CMV, dengue, GBV, herpes simplex, hepatitis A, hepatitis B, hepatitis non A-non B, hepatitis C, influenza, lassa fever, parainfluenza, pneumonia, parvovirus, rotavirus, and RSV.

16. The method of claim 9, wherein the LFA-3 is coupled directly to the immunogen.

17. The method of claim 9, wherein the mammal is also inoculated with (3) a co-adjuvant consisting of a polypeptide or antibody that binds to a surface antigen on T-lymphocytes.

18. The method of claim 15, wherein the co-adjuvant is anti-$T11_1$, anti-$T11_2$ or anti-$T11_3$, or fragments thereof recognizing an epitope of CD2.

19. A therapeutic treatment for bacterial or viral diseases comprising administering to a mammal exposed to the disease an amount of a combination of (1) LFA-3 and (2) anti-$T11_1$, anti-$T11_2$, anti-$T11_3$, fragments thereof, or a combination thereof, effective to enhance proliferation of the T-lymphocytes activated by the agent causing said disease.

FIG. 1

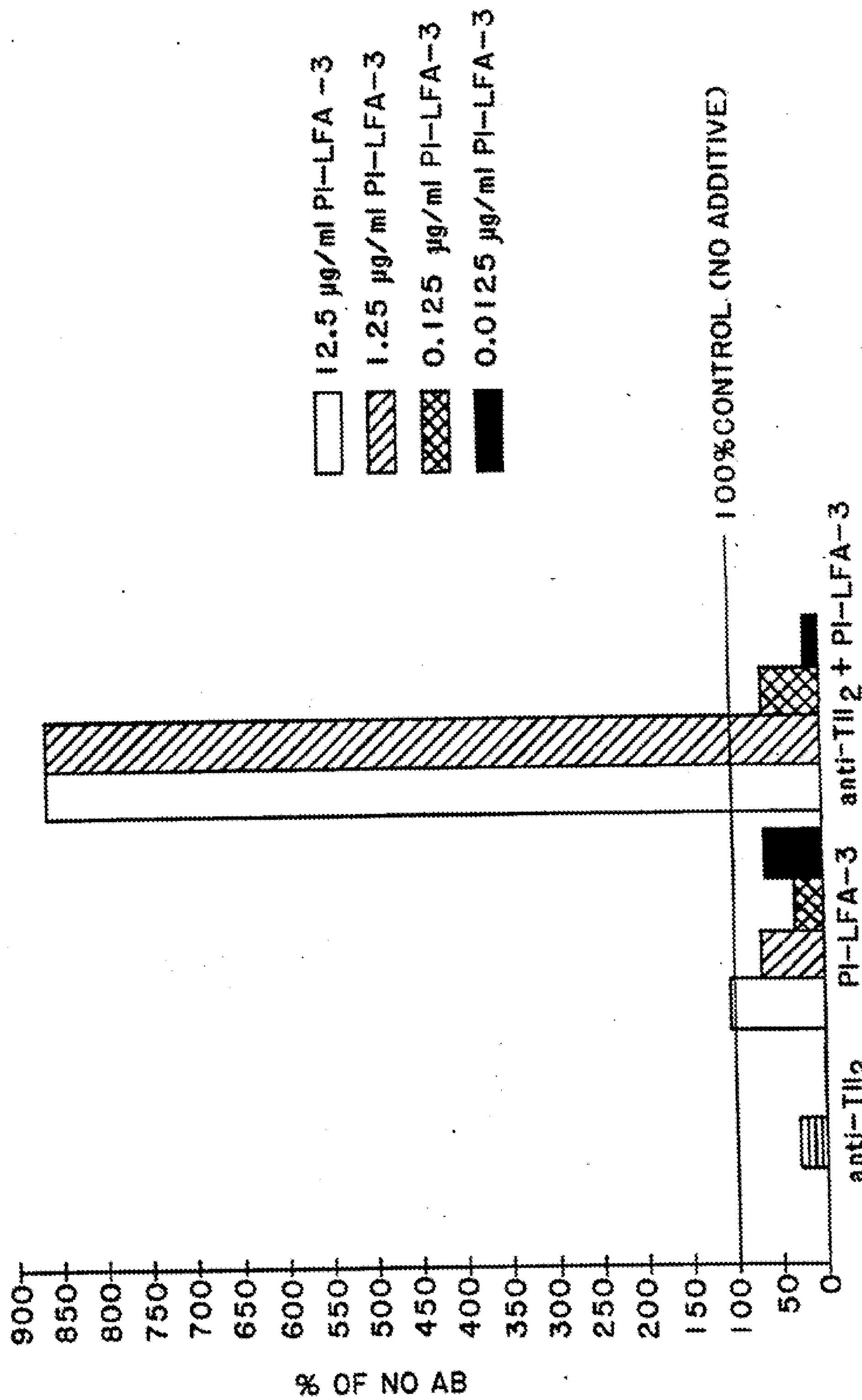
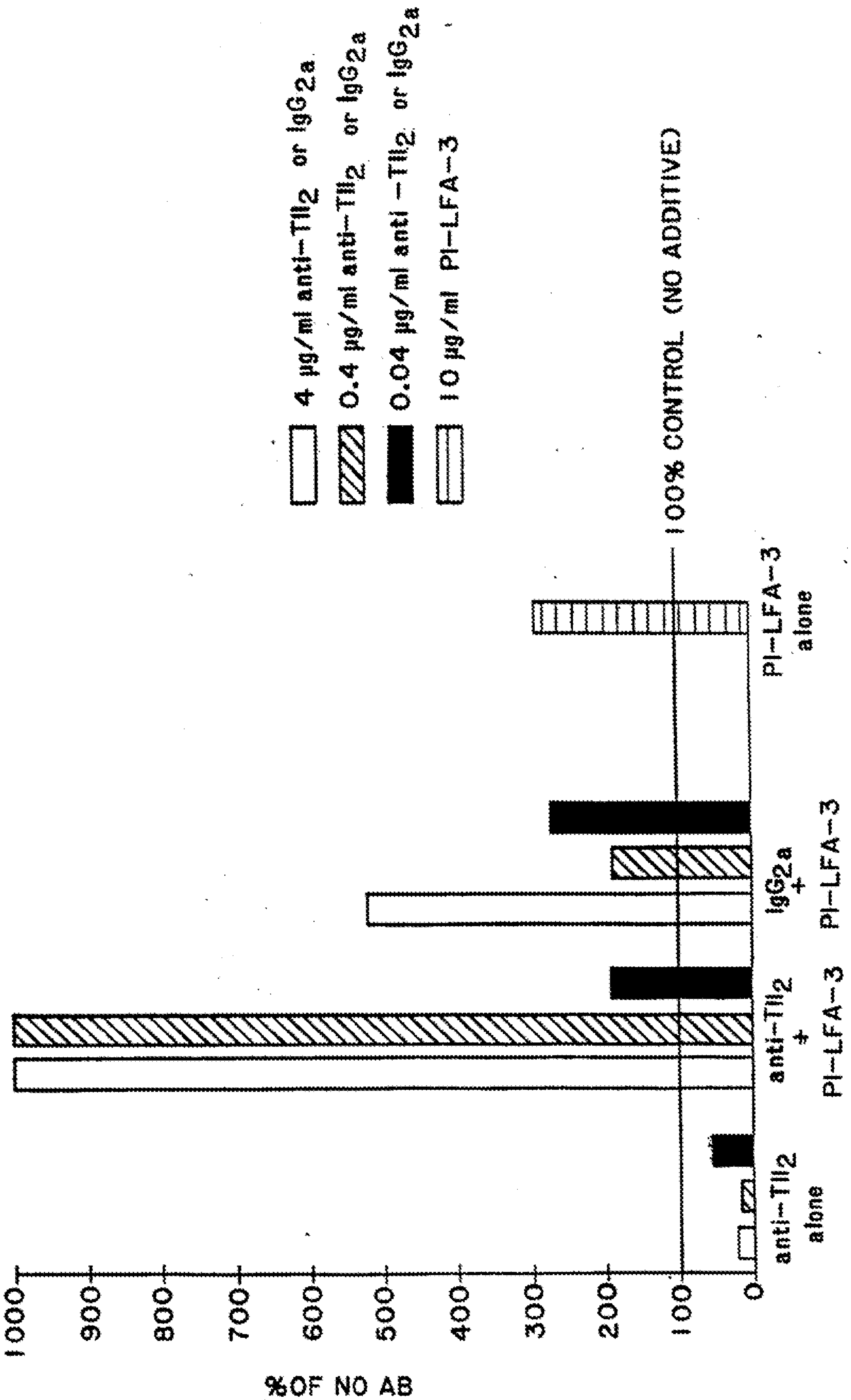

12.5 µg/ml PI-LFA-3
1.25 µg/ml PI-LFA-3
0.125 µg/ml PI-LFA-3
0.0125 µg/ml PI-LFA-3
100% CONTROL (NO ADDITIVE)
anti-TII2 alone
PI-LFA-3 alone
anti-TII2 + PI-LFA-3
% OF NO AB
0
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900

FIG. 2


4 μg/ml anti-TII2 or IgG2a
0.4 μg/ml anti-TII2 or IgG2a
0.04 μg/ml anti-TII2 or IgG2a
10 μg/ml PI-LFA-3
100% CONTROL (NO ADDITIVE)
%OF NO AB
1000
900
800
700
600
500
400
300
200
100
0
anti-TII2 alone
anti-TII2 + PI-LFA-3
IgG2a + PI-LFA-3
PI-LFA-3 alone

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/00508

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/02, 39/00

U.S.Cl.: 424/88; 514/12

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/88; 514/12

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Automated Patent System and DIALOG Databases.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P,Y	US,A, 4,956,281 (WALLNER et al.) 11 September 1990. see entire document.	1-19
Y	BIOSIS, Philos. Trans. R. Sci. Lond. B. Biol. Sci., Volume 323, Issued 1989. M.J. Crumpton "T-lymphocyte Recognition and Response to Antigen", Page 519. See abstract, accession number 90075684.	1-19
Y	BIOSIS, Immunogenetics, Volume 28(4), issued 1988, W.A. Sewell, et al., "The human LFA-3 gene is located at the same chromosome band as the gene for its receptor CD2," Pages 278-282. See abstract, accession number 87004781.	1-19
Y	BIOSIS, Proc. Natl. Acad. Sci., Volume 85(4), Issued 1988, B.E. Bierer, et al., "Expression of the T-cell surface molecule CD2 and an epitope-loss CD2 mutant to define the role of lymphocyte function associated antigen 3 LFA-3 in T-cell activation," Pages 1194-1198. See abstract, accession number 85110876.	1-19

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
04 March 1991	04 JUN 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Thomas M. Cunningham Thomas M. Cunningham, Ph.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	BIOSIS, Eur. J. Immunol., Volume 19(4), issued 1989, B.F. BIERER, et al., "A monoclonal antibody to LFA-3 the CD2 ligand specifically immobilizes major histocompatibility complex proteins.", pages 661-666. See abstract, accession number 88038562.	1-19
Y	BIOSIS, J. Exp. Med., Volume 166(4), issued 1987, B.P. Wallner, et al., "Primary Structure of Lymphocyte Function-Associated Antigen 3 LFA-3 the Ligand of the T Lymphocyte CD2 Glycoprotein." Pages 923-932. See abstract, accession number 85012425.	1-19
Y	BIOSIS, Immunol., Volume 139(8), issued 1987, S.M. Denning, et al., "Monoclonal antibodies to CD2 and Lymphocyte Function-Associated Antigen 3 inhibit human thymic epithelial cell-dependent mature thymocyte activation". Pages 2573-2578. See abstract, accession number 85004878.	1-19
Y	BIOSIS, Proc. Natl. Acad. Sci., Volume 84(19), issued 1987, Y. Takai, et al., "Direct evidence for a receptor ligand interaction between the T cell surface antigen CD2 and Lymphocyte Associated Antigen 3, Pages 6864-6868. See abstract, accession number 85004802.	1-19
Y	BIOSIS, J. Immunol., Volume 138(2), issued 1987, L.W. Vollger, et al. "Thymocyte binding to human thymic epithelial cells in inhibited by monoclonal antibodies to CD2 and LFA-3 antigens. Pages 358-363. See abstract, accession number 83075839.	1-19
Y	Medline, Surv. Immunol. Res., Volume 3(1), issued 1984, A.M. Krensky, et al., "Human lymphocyte function-associated antigens". Pages 39-44. See abstract, accession number 84195633.	1-19